Electrophoresis and isoelectric focusing of whole cell and membrane proteins from the extremely halophilic archaebacteria

Helga Stan-Lotter, Frank J. Lang, Jr. & Lawrence I. Hochstein

NASA Ames Research Center, Moffett Field, California 94035, USA

NASA/CR – 89 — 206073

The isoelectric points of most proteins from the extremely halophilic archaebacteria are between 4.0 and 4.65 which agrees with the generally high content of glutamic and aspartic acid in proteins from halobacteria. The subunits from two purified halobacterial membrane enzymes (ATPase and nitrate reductase) behaved differently with respect to isoelectric focusing, silver staining and interaction with ampholytes. Differential behavior was also observed in whole cell proteins from *Halobacterium saccharovorum* regarding resolution in two-dimensional gels and silver staining. We propose that these differences reflect the existence of two classes of halobacterial proteins, one resembling non-halophilic proteins, and the other possessing unique properties that may be related to salt dependence.

Introduction

Extremely halophilic bacteria require from 2 to 4 M NaCl for growth and many grow in medium that is saturated with respect to NaCl (Kushner 1978). In addition, they contain a high intracellular salt concentration, with K⁺ > Na⁺ (Bayley & Morton 1978) which is reflected by the presence of proteins that are stabilized by relatively high concentrations of monovalent cations (Lanyi 1974). One consequence of this dependence for high concentrations of monovalent cations is that relatively few halobacterial proteins have been purified since many methods of protein purification are performed at low ionic strength. The proteins which have been purified (for recent reviews see Eisenberg & Wachtel 1987; Russell 1989) generally require high concentrations of salt for stability and biological activity. However, a few halobacterial proteins are known to be stable, at least to some extent, in the absence of high ionic strength (May & Dennis 1987; Russell 1989).

At present, there is incomplete understanding of the interactions between proteins and a high salt environment (Eisenberg & Wachtel 1987). It has been known for some time that the halophilic proteins have an excess of acidic over basic amino acids and a deficiency of hydrophobic amino acids (Reistad 1970; Lanyi 1974). With some protein sequences now available, amino acid substitutions between halophilic and their nonhalophilic counterparts can be evaluated on a quantitative basis (Kimura & Kimura 1987; Kimura *et al.*, 1989; May *et al.*, 1989).

However, a general explanation of how the amino acid composition affords the unusual tolerance of or even dependence on high ionic strength of halophilic proteins cannot yet be given.

Polyacrylamide gel electrophoresis and isoelectric focusing are powerful tools for analyzing proteins at the molecular level. These methods have not been widely applied to halobacterial proteins. There are several technical problems. Electrophoretic procedures are not compatible with the presence of high concentrations of salt. About 0.1 M salt or even less interferes with separation in polyacrylamide gel electrophoresis (Maurer 1971), isoelectric focusing with carrier ampholytes (Vesterberg 1975) or with immobilized gradients (Righetti *et al.*, 1988). Thus, electrophoretic separation of halobacterial proteins under non-denaturing conditions is in most cases not possible. The acidic nature of halobacterial proteins can lead to additional difficulties. These proteins may possess as much as 20% molar excess of acidic amino acids over basic amino acids (Reistad 1970; Bayley & Morton 1978). Some acidic proteins behave anomalously during electrophoresis in the presence of sodium dodecyl-sulfate probably because of reduced binding of sodium dodecylsulphate (SDS) (Dunker & Rueckert 1969; Izotova *et al.*, 1983; Lechner & Sumper 1987). This results in a reduction of expected mobility and leads to an overestimation of molecular masses. Acidic proteins also do not readily electrofocus (Stenman 1975; Radola 1975; Green *et al.*, 1986).

We are engaged in the investigation of halobacterial membrane proteins. One such enzyme, the ATPase from *Halobacterium saccharovorum*, is strictly salt dependent for its activity and stability (Altekar *et al.*, 1984; Kristjansson & Hochstein 1985). We obtained preliminary results for the two major subunits of this enzyme following SDS gel electrophoresis and isoelectric focusing, which we reported earlier (Stan-Lotter & Hochstein 1989). The most obvious differences to nonhalophilic proteins were streaking in isoelectric focusing gels of the O'Farrell type (1975) and the nearly complete absence of silver stainability. We have also purified the nitrate reductase from the membranes of *H. denitrificans*. This enzyme is active and stable in the absence of high concentrations of salt (Hochstein and Lang, manuscript in preparation).

In order to investigate suitable conditions for electrophoresis and isoelectric focusing of proteins from the extremely halophilic archaebacteria, we examined various cell fractions, whole cell proteins and the two purified enzymes mentioned above. We show that an acidic protein, soy bean trypsin inhibitor, which, following chemical modification, had lower isoelectric points than most halobacterial proteins, is readily focused. Results with the halobacterial proteins following electrophoresis and isoelectric focusing lead us to suggest that

they may fall into discrete classes. We discuss a possible correlation of our observations with the salt dependence of halobacterial proteins

Materials and methods

Chemicals

The following materials were supplied by the indicated companies. Sigma Chemical Company: Tween 40, Tween 80, soybean trypsin inhibitor, bovine serum albumin. Pierce: Tween 20, Brij-35, BCA Protein Assay Reagent. Pharmacia-LKB: Pharmalyte pH 2.5 – 5, pH 4.2 – 4.9 and pH 3 – 10; LKB Ampholine pH 3.5 – 5. Serva: Servalyt pH 4 5 and pH 3 10. Calbiochem: Zwittergent, 3-((3-cholamidopropyl)-dimethylammonio-1-propanesulfonate (CHAPS), N,N-bis-(3-D-glucon-amidopropyl)-cholamide (Big CHAP). BioRad: BioRad Protein Assay Reagent, γ-globulin, Silver Stain Kit, isoelectric focusing (IEF)-standards, urea, Triton X-100, SDS. Roche Diagnostics: Fluorescamine (Fluram). Eastman Kodak Co.: Sodium deoxycholate.

Preparation of cell fractions from Halobacterium saccharovorum

The membrane fraction of *Halobacterium saccharovorum* (ATCC 29252) was prepared by disrupting cells in a French pressure cell and sedimenting the membranes by centrifugation as described by Kristjansson and Hochstein (1985) The resulting supernatant was used as the source of the cytoplasmic fraction These fractions were stored at 22°C in the dark. Whole cells were lysed by incubating them at 22°C in deionized water at a concentration of 50 mg (wet weight) per ml. After ~ 2 h, the viscosity of the lysed cell suspension was considerably reduced and the lysed material was immediately stored at – 20°C.

Purification of halobacterial enzymes and subunits

The halobacterial ATPase was prepared from the membrane fraction of *H. saccharovorum* following solubilization with Zwittergent and sodium deoxycholate (Hochstein et al. 1987; Stan-Lotter & Hochstein 1989). The major subunits of the ATPase (I and II) were electroeluted following SDS gel electrophoresis as described previously (Stan-Lotter & Hochstein 1989). The dissimilatory nitrate reductase from the membranes of *Halobacterium denitrificans* (Tomlinson et al., 1986) was purified following solubilization in the absence of detergents using anion exchange, hydroxylapatite and gel filtration chromatography (Hochstein and Lang, manuscript in preparation).

Polyacrylamide gel electrophoresis

One-dimensional slab SDS gel electrophoresis was performed according to Laemmli (1970). Isoelectric focusing was performed in a vertical slab gel apparatus (Stan-Lotter & Bragg 1986a) with the following modifications: the gels were 0.5 mm thick and the voltage was set to 1000 V. The IEF gel system used was the one described by O'Farrell (1975) and modified, where indicated, by replacing Triton X-100 with various detergents or using

different concentrations of ampholytes. Two-dimensional gel electrophoresis was performed either by O'Farrell's method (1975) using gel strips instead of tube gels for the IEF step or by carrying out SDS gel electrophoresis in the first dimension and IEF in the second dimension (Stan-Lotter & Bragg 1986a). The pH gradient of IEF gels was determined after completion of the run by eluting 1 cm³ gel slices with 1 ml of deaerated water for 1 h and measuring the pH of the solution. Gels were stained with Coomassie Blue (Fairbanks et al., 1971) or silver (BioRad silver stain kit). Periodic-acid Schiff reagent (PAS) (Fairbanks et al., 1971) was used to detect glycoproteins. Permanent records of the stained gels were made with Electrophoresis duplicating paper (Kodak) or photographed using Polaroid film 57 and an orange filter. Initially, contact prints were made using wet gels as recommended by the manufacturer. We find it preferable to first dry the gels on a cellophane membrane.

Other methods

Proteins were labelled with fluorescamine as described earlier (Stan-Lotter & Hochstein 1989). Modification of proteins with succinic anhydride was carried out by the procedure of Hollecker and Creighton (1980). Protein concentrations were determined by the methods of Bradford (1976), Smith et al. (1985), or Lowry et al. (1951) Bovine serum albumin or γ-globulin were used as standards.

Results

SDS gel electrophoresis of halobacterial whole cell proteins

Figure 1 shows an electrophoretogram obtained from early stationary phase cells of *H. saccharovorum*. The cells were lysed in water and the proteins in the lysate were treated with SDS sample buffer (Laemmli 1970). A wide range of molecular species were present and appeared to be almost uniformly distributed between M_r's of 15×10^3 and 200×10^3. Lanes 1 and 2 (Figure 1) were stained with Coomassie Blue. Lanes 3 and 4 are the same gels which, following staining with Coomassie Blue, were incubated in 25% isopropanol-10% acetic acid, to remove the dye, and subsequently stained with silver. Silver staining did not reveal the presence of additional peptides or enhance the detectability of those proteins which had stained with Coomassie Blue. On the contrary, several proteins, which reacted intensely with Coomassie Blue, stained weakly with silver (indicated by arrows). Several diffuse and slowly migrating bands as well as some which were retained in the stacking gel stained more strongly with silver. Some appeared to be glycoproteins since they also stained with PAS reagent (not shown).

IEF of membrane and cytoplasmic fractions

Virtually all of the membrane proteins from *H. saccharovorum* clustered at the anodic end indicating isoelectric points below 4.5 (Figure 2, lane 1). Most of the cytoplasmic proteins also migrated towards the anodic end, however, a few bands with isoelectric points up to 5.7 were visible (Figure 2, lanes 2, 3). When isoelectric focusing was carried out within a narrower pH gradient
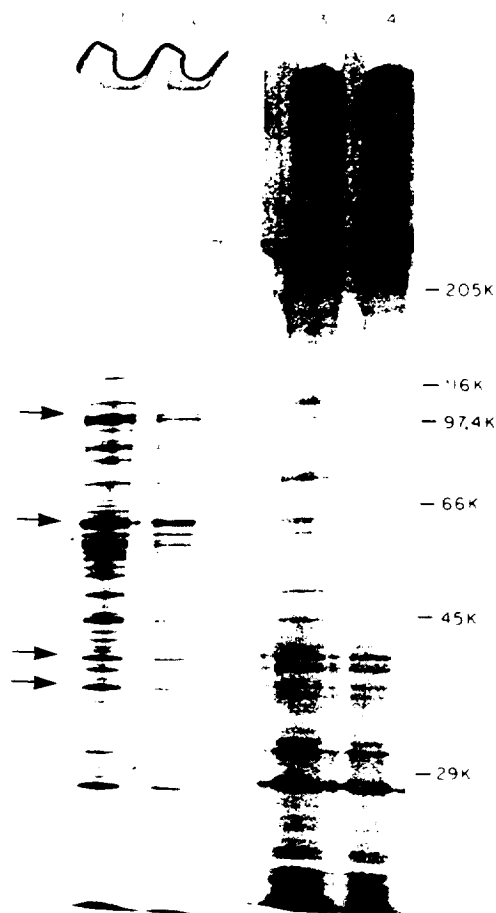
Figure 1   SDS polyacrylamide gel electrophoresis of total cell proteins from *H. saccharovorum*. Cells from *H. saccharovorum* were lysed as described in Methods. Acrylamide concentration was 11% T, 2.6% C. Gels were stained with Coomassie Blue (lanes 1, 2) and with silver (lanes 3, 4). Molecular mass markers are indicated on the right. Arrows point to some proteins which stained well with Coomassie, but only weakly with silver. Lanes 1, 3: 3.7-4 µg protein, lanes 2, 4: 18.7 µg protein.

Figure 2   Isoelectric focusing of halobacterial proteins in wide and narrow pH gradients. Samples were prepared from *H. saccharovorum* (lanes 1 to 5) and *H. denitrificans* (lane 6) as described in Methods and are as follows. Membrane fraction (lanes 1, 4), cytoplasmic proteins (lanes 2, 3, 5), purified nitrate reductase (lane 6). The IEF gels contained 8 M urea, 4% acrylamide, 2% Triton X-100 and 2% Pharmalytes pH 3–10 (lanes 1 to 3) or pH 2.5 to 5 (lanes 4 to 6), respectively. The approximate pH gradients are indicated. Gels were stained with Coomassie Blue. Protein concentrations were: lane 1, 51 µg, lane 2, 21 µg, lane 3, 26 µg, lane 4, 26 µg, lane 5, 24 µg, lane 6, 4.5 µg.
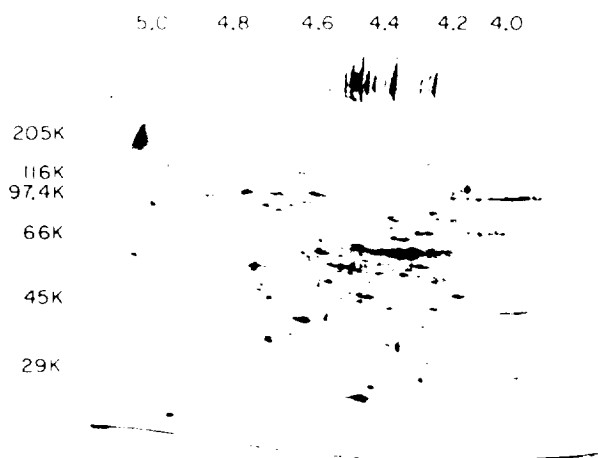

Figure 3   Two-dimensional electrophoresis of total cell proteins from *H. saccharovorum*. The approximate pH gradient established in the first dimension is shown on the top, together with an IEF strip containing ~ 98 µg of total cell protein. Molecular mass standards are indicated alongside the second dimension SDS gel. 152 µg of protein were applied. Gels were stained with Coomassie Blue.

(3.5 to 5.0), most of the proteins equilibrated between approximately pH 4.0 and 4.6 (Figure 2, lanes 4, 5). When isoelectric focusing was carried out either in the absence of urea or in the presence of 2 M urea, the proteins precipitated in the sample wells. This was true for membrane and soluble halobacterial proteins as well as the isolated halobacterial membrane enzymes described below (gels not shown). Proteins from non-halobacterial sources (IEF standards) were successfully focused in these gels (not shown).

Two-dimensional gel electrophoresis of halobacterial whole cell proteins

Figure 3 shows a two-dimensional gel of the total cell proteins from *H. saccharovorum*. The first dimension was a strip from an IEF slab gel (upper part of Figure 3). The two-dimensional gel showed numerous well-resolved spots over the whole range of molecular masses. Very little microheterogeneity of proteins, as revealed by spot trains, was apparent. However, several proteins exhibited horizontal streaking, most notably in the acidic region of the gel. In addition, some of the higher molecular mass proteins tended to streak. This pattern was reproducible

whether equilibration times for the IEF dimension in SDS sample buffer (Laemmli 1970) were as short as 10 min or up to 40 min. The blurred spot in the upper left corner of the SDS dimension stained also with the PAS reagent (not shown) and most likely represented the cell surface glycoprotein, which may be similar to the one from *Halobacterium halobium* (Lechner & Sumper 1987).

IEF of isolated halobacterial membrane enzymes and their subunits

(1) Nitrate reductase   The nitrate reductase from the membranes of *H. denitrificans* was analyzed on a standard

IEF gel (see Methods) following dissociation with 4 M urea and 2% Triton X-100 (Figure 2, lane 6). The main component was a protein with an apparent isoelectric point of 4.65, which migrated as a double band. This protein represented a subunit of a M_r of 120×10^3, as could be deduced from a two-dimensional gel with a similar IEF strip as the first dimension (gel not shown). Other subunits of the nitrate reductase were more acidic and had smaller molecular masses. All subunits of the nitrate reductase stained well with silver, in contrast to other proteins from halobacteria (see Figure 1).

(2) Adenosine triphosphatase. The membrane-bound ATPase from *H. saccharovorum* contains two major subunits of M_r of 87×10^3 and 60×10^3, subunits I and II, respectively (Hochstein *et al.*, 1987; Stan-Lotter & Hochstein 1989). A two-dimensional system with SDS PAGE as the first and IEF as the second dimension has been described which yields sharply focused bands of various native and chemically modified eubacterial proteins (Stan-Lotter & Bragg 1986 a–d). The same system was not effective with either subunits I and II of the halobacterial ATPase (Figure 4). Both polypeptides migrated into the acidic region of the IEF gel, but did not focus, even when 30 000 Volthours (instead of the usual 9000) were applied. Similar patterns were obtained with IEF gels containing different concentrations of Triton X-100 (from 1 to 4%), or carrier ampholytes from different commercial suppliers. Degradation during electrophoresis was ruled out as a cause of streaking since excised IEF gel strips containing the ATPase subunits showed single bands when run in an SDS gel (not shown). One-dimensional IEF in Triton-containing gels of subunits I and II, which were largely freed of SDS by electroelution, gave essentially similar patterns as those shown in Figure 4.

Acidic proteins can be difficult to electrofocus due to lack of suitable carrier ampholytes capable of establishing pH gradients in the required range and to prolonged focusing times (see Stenman 1975; Green *et al.* 1986 and references therein). We examined an acidic non-halobacterial protein, soybean trypsin inhibitor, which was chemically modified to incorporate up to 22 additional negative charges. The modifying reagents were succinic anhydride or 4-phenylspiro(furan-2(3H), 1'-phthalan-3,3'-dione (fluorescamine). Both react with amino groups and impart two negative charges per modified group into a protein. Figure 5 shows an IEF gel of soybean trypsin inhibitor, which was sequentially modified with increasing concentrations of succinic anhydride (panel a) or fluorescamine (panel b). Modification of the protein by succinic anhydride resulted in the appearance of new bands with additional negative charges. A total of 11 such bands were visible, as seen in the mixture of all modified species (panel a, lane 8) which corresponded to the 10 ε-aminogroups of lysyl residues and one amino-terminal group of the trypsin inhibitor (Koide *et al.*, 1972). The approximate isoelectric point of the fully modified species was 3.8, which was lower than that of most halobacterial proteins (see Figures 2, 3). Since this very acidic protein could be focused into sharp bands, the acidic nature of a protein, *per se*, is not the likely cause of streaking in IEF gels. Interestingly, fluorescamine-modified trypsin inhibitor yielded slightly more diffuse bands following IEF (panel b, lanes 4 and 5) than the succinic anhydride modified
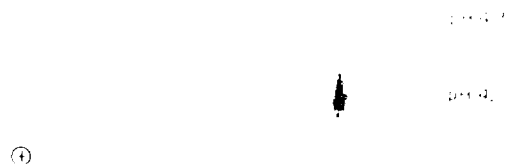

Figure 4   Isoelectric focusing of the two major subunits of the ATPase from *H. saccharovorum* in the presence of Triton X-100. Subunits were separated first by SDS PAGE and excised from the unstained gels. Following equilibration in 8 M urea, 4% Triton X-100, 1% Pharmalyte pH 2.5–5, the protein containing gel slices were applied on a vertical isoelectric focusing gel consisting of 8 M urea, 2% Triton X-100, 4% acrylamide and 2% Pharmalyte pH 2.5 to 5. Gels were stained with Coomassie Blue. The approximate pH gradient is indicated. Lane 1, subunit II, ~ 10 µg, lane 2, subunit I, ~ 12 µg.
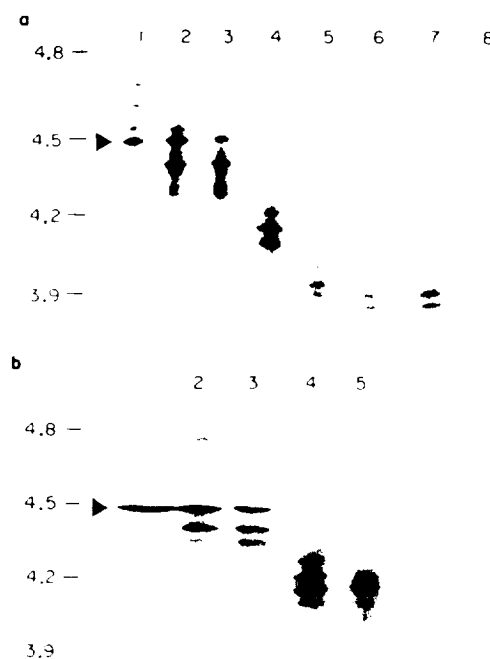

Figure 5   Conversion of amino groups of soybean trypsin inhibitor into acidic groups by chemical modification. Increasing amounts of succinic anhydride (panel a) or fluorescamine (panel b) were added to soybean trypsin inhibitor. The resulting species with increased negative charges were separated on an IEF gel containing 8 M urea, 2% Pharmalyte pH 2.5 to 5, 4% acrylamide, 2% Tween 40. Gels were stained with Coomassie Blue. Lanes 1, a and b, show the original unmodified protein, the main component with an approximate isoelectric point of 4.5 is indicated by the arrows. In panel a, lane 8, a mixture of all modified species, including the untreated protein (arrow) was applied. The ratios of succinic anhydride to protein (w:w) were 0.14, 0.26, 0.94, 1.74, 2.24, and 2.94, respectively (a, lanes 2 to 7); the ratios of fluorescamine to protein were 0.0008, 0.016, 0.083, and 0.166, respectively (b, lanes 2 to 5). Protein content was 21 µg per lane.

protein. It appeared that the chemical composition of the modified protein influenced its behavior during electrofocusing.

The effect of detergents on electrofocusing of the halobacterial ATPase subunits was studied by replacing

Triton X-100 with a variety of nonionic or zwitterionic detergents. When CHAPS or BigCHAP were used, subunit I focused well at an isoelectric point of about 4.1 whereas subunit II still streaked (not shown). Sharp focusing of both subunits was observed only with the Tween series (Tween 20, 40, 80) of detergents. We employed Tween 40 at a final concentration of 2%, as had been briefly reported earlier (Stan-Lotter & Hochstein 1989). Protein-protein interactions can result in streaking during IEF. Our results suggest that if such interactions take place between halobacterial ATPase subunits, not all detergents are effective counteragents.

Streaking of both ATPase subunits even in the Tween 40-containing IEF gels was observed, when higher protein loads were used. A possible, if unusual, explanation for this phenomenon would be an interaction between ampholytes and proteins. In order to test this possibility, IEF was performed in gels, which contained decreasing amounts of ampholytes, but same amounts of proteins. As shown in Figure 6, there was a correlation between streaking and ampholyte concentration. Both ATPase subunits showed streaking at ampholyte concentrations of 2% (Figure 6, lanes 1 and 3). Subunit I focused sharply in gels containing 0.7% ampholyte (Figure 6, lane 2) whereas subunit II still exhibited streaking (Figure 6, lane 4). Subunit II focused sharply in IEF gels containing 0.35% ampholyte (Figure 6, lane 5).

Discussion

The bulk proteins from halobacteria possess a large excess of acidic over basic amino acids (Reistad 1970) as do most of the ribosomal proteins (Matheson 1985; Kimura & Kimura 1987; Kimura et al., 1989) and other halobacterial proteins which have so far been analysed (Mevarech et al., 1977; Danson et al., 1986; Lechner & Sumper, 1987; Stan-Lotter & Hochstein 1989). Thus, the observed low isoelectric points of the bulk membrane and soluble proteins (Figures 2, 3) correlated with the known amino acid compositions of halobacterial proteins. A

Figure 6 Isoelectric focusing of the two major subunits of the ATPase from *H. saccharovorum* at different concentrations of ampholyte. Isoelectric focusing was performed following SDS gel electrophoresis as described in the legend to Figure 4, except that Tween 40 was used instead of Triton X-100. The IEF gel contained different concentrations of Pharmalyte pH 2.5 to 5. Gels were stained with Coomassie Blue. Lanes 1, 2, subunit I; lanes 3 to 5, subunit II. Ampholyte concentrations: lanes 1, 3, 2%, lanes 2, 4, 0.7%, lane 5, 0.35%. Protein per lane: 1, 2, 9 × 5 µg, 3 to 5, 8.5 µg

number of halobacterial proteins from whole cell extracts as well as the two major subunits of the membrane ATPase from *H. saccharovorum* either stained weakly or did not stain with silver. This was not correlated with the origin of the enzyme, since the nitrate reductase, which is a membrane-bound enzyme, stained very well with silver (not shown). There might be a correlation with the low content of cysteinyl, lysyl and methionyl residues (Stan-Lotter & Hochstein 1989) which are the amino acids implicated in silver grain nucleation (Merril & Pratt 1986).

Streaking during isoelectric focusing is essentially a technical problem which has been associated with insufficient focusing time, unsuitable carrier ampholytes, and formation of precipitates due to protein-protein interactions (for an extensive discussion of the latter phenomenon see Hjelmeland and Chrambach 1981). We were able to exclude the first two causes in the case of the halobacterial ATPase subunits since increasing focusing times did not eliminate the problem, and the gel system we used resulted in good separation of very acidic proteins (Figure 5). Moreover, streaking was not observed with the subunits from the halobacterial nitrate reductase. The standard IEF gel according to the formulation of O'Farrell (1975) with a final detergent concentration of 2% has been widely used for complex protein mixtures from different sources and proved suitable for eliminating precipitates due to protein-protein interactions. Thus, the persistent aggregations of the halobacterial ATPase subunits and some whole cell proteins under these conditions were puzzling, particularly in view of the high content of hydrophilic and the marked deficiency of hydrophobic amino acids in halobacterial proteins (Lanyi 1974) and their relatively high solubility (e.g. about 0.12 mg ml^{-1} in 7.5 mM ammonium bicarbonate for purified ATPase subunit II; Stan-Lotter, unpublished observation). Isoelectric focusing of the halobacterial ATPase subunits suggested interactions between protein and ampholytes as a further phenomenon which leads to streaking. Although carrier ampholytes were reported to interact with acidic substances such as heparin (Righetti & Gianazza 1978), reactions with proteins to the point of precipitation are unusual. Indeed, carrier ampholytes are often added in immobilized pH gradients to promote protein solubilization (Rimpilainen & Righetti 1985; Righetti et al., 1988). However, protein-ampholyte interaction cannot be a common property of the proteins from extremely halophilic bacteria since the nitrate reductase, as well as numerous other proteins from a halobacterial cell extract, focused in a standard IEF gel.

Our data suggest the possibility that halobacterial proteins may fall into two classes. One resembles the proteins from non-halophilic organisms with respect to stainability with silver and isoelectric focusing in Triton X-100 containing gels. The other class, which either fails to stain or only weakly stains with silver, reacts with ampholytes to form a precipitate during IEF, which can be dispersed by several detergents, but not Triton X-100. There may be subtler differences within members of this class, as indicated by the ready focusing of ATPase subunit I, but not of subunit II, in IEF gels containing various detergents. Since halobacterial proteins are acidic and can reasonably be assumed to be similar with respect to their overall amino acid composition (Bayley & Morton 1978), other features, perhaps structural, may account for the observed differences. The possibility of as

yet unidentified substructures in halophilic proteins had been suggested recently to explain their unusual properties (Eisenberg & Wachtel 1987).

The proposed differentiation of halobacterial proteins correlates with the salt-dependence of the two halobacterial membrane enzymes which we analyzed. Thus, the nitrate reductase, which is active and stable in the absence of high concentrations of salt (Hochstein and Lang, manuscript in preparation) would fall into the class resembling the non-halophilic proteins, whereas the ATPase is representative of those proteins which require high concentrations of salt. These conclusions are to be considered as a working hypothesis which is amenable to experimental verification.

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